Damaging nsSNPs in ULK1 gene and Susceptibility to Hepatitis B infection

SYNOPSIS

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1. Introduction

Worldwide, hepatitis infection is intensifying at an alarming pace and demands emergent attention. According to W.H.O. 2017 report, approximately 240 million people are chronically infected with hepatitis B around the world[1] and an estimate of 6.5 million people die every year due to complications caused by hepatitis B virus (HBV) infection[2]. With the highest prevalence in sub-Saharan Africa and East Asia, around 5–10% of the adult population is chronically infected. An assessment states that 2–7% of the general population is chronically infected in the Middle East and the Indian subcontinent[3]. Till date, India constitutes 17.6% of the world's population and accounts for 10–15% of the worldwide HBV burden **[4]**.

Human HBV is the member of the Hepadnaviridae family whose natural hosts are humans, apes, and birds. Currently, this virus family is divided into 2 genera: Avihepadnavirus (infect birds) and Orthohepadnavirus(infect mammals). Basically, the human HBV genome is a 3.2 kb circular and partially double-stranded DNA that includes four overlapping open reading frames (ORFs) encoding the surface (preS1, preS2, and S), core (C and pre-core), polymerase (P), and X proteins [5]. Hepatitis B surface antigen (HBsAg), a foremost indicator of HBV prevalence is commonly used for evaluating HBV endemicity around the world. The mode of transmission of this virus from a person is usually through blood or body fluids. Despite effective vaccinations, HBV infections are still very common globally as the virus replicates by reverse transcription using a viral polymerase lacking proof-reading ability which results in the emergence of mutant viruses that remain undetected by host immunity or viral therapeutic agents. Other mutations result in a change in the HBV surface antigen, resulting in a loss of detection by some diagnostic assays [6].

From decades, extensive evidence suggest an important role of hepatitis B virus X protein, a small soluble cytoplasmic protein in the host cells, which modulate gene expression or intracellular signal pathway through interaction with either transcriptional machinery or signaling components involved in cell proliferation, apoptosis, and DNA repair [7, 8]. Importantly, it has also been shown that Hepatitis B virus uses autophagy machinery for its own survival and proliferation via its X protein [9-11]. The clinical presentation of the disease in patients with chronic hepatitis B infection depends on several host and viral factors. Out of these, autophagy machinery is one of the chief factors contributing to the progression and replication of HBV [12].

Autophagy, an evolutionary conserved and the strongly regulated catabolic process is primarily involved in the degradation of cellular components through lysosomal machinery. This process is indispensable in many cellular events including differentiation, survival, development, and homeostasis [13]. From a total of 31autophagy related genes (ATG) identified till date[14], *ATG1/ULK1*(uncoordinated 51-like kinase 1) complex plays a central role in autophagy. It works by integrating signals from the upstream mechanistic target of rapamycin (mTOR) and 5' AMP-activated protein kinase (AMPK) and transduces them to the downstream autophagy pathway. While autophagy occurs at the basal levels under standard conditions, it is often activated in response to cellular stresses such as endoplasmic reticulum (ER) stress, nutrient starvation, hypoxia, and pathogen infection. Upon these stress conditions, the cell utilizes autophagy either to redistribute its resources to tide over the period of stress or to degrade injurious components (such as, damaged mitochondria or invading pathogens) via lysosomal degradation[15].

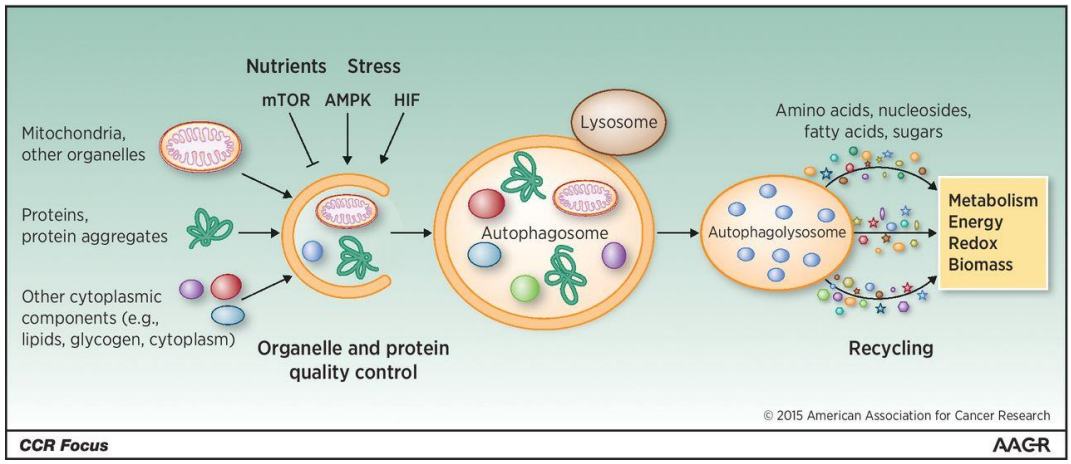

Figure1. Overview of mammalian autophagy (White E *et al*. 2015)

Recently, Sir *et al*. clearly revealed the enhancement of HBV DNA replication by autophagy [16], they further confirmed the role of autophagy in the production of HBV virions *in vivo* using HBV transgenic mice and also the formation of autophagosome is essential for HBV DNA synthesis in the cytoplasm [17].

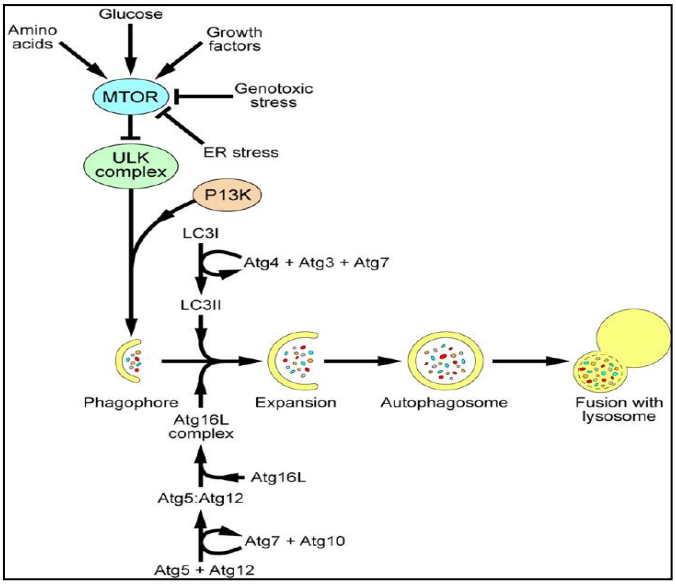

Figure 2. Pathway involved in autophagy (Maier HJ *et al*. 2012)

In particular, *ULK1* an ortholog of yeast autophagy-related gene (*ATG1*), is a critical mammalian kinase regulator of autophagy [18, 19]; as evident from studies depicting the correlation between autophagy induction and HBV expression [20, 21]. In mammals, *ULK1* is the principal gene of autophagy pathway, which gets activated first and further initiates autophagy by regulating the downstream genes. Till date, the genetic variations in *ULK1* have been studied with an only handful of diseases which includes*Mycobacterium tuberculosis* infection, Behçet's disease (BD) and Vogt-Koyanagi-Harada (VKH) syndrome, congenital heart malformation, and Crohn's disease [22-26]. Despite of strong association studies of autophagy with hepatitis B infection available in literature, there are no genetic association studies reported between autophagy initiating gene, *ULK1* and Hepatitis B. Therefore, assuming the hypothesis that *ULK1* controls the induction of autophagy by forming complex with *ATG13*, *FIP200*, and *ATG10* and can be associated with Hepatitis B infection. In this study, we analyzed the four predicted damaging non-synonymous SNPs (nsSNPs) by *in-silico* analysis and two earlier reported single nucleotide polymorphisms (SNPs) i.e. rs3923716 (G/T) and rs12303764 (G/T) in *ULK1* known to be associated with Crohn's Disease [26, 27] in a New Zealand Population. Mainly, our study focuses on the association of polymorphisms in *ULK1* gene and their susceptibility to HBV infection in North Indian Population.

2. Objectives of the study

Objective 1:

To predict the damaging nsSNPs in *ULK1* gene through *in-Silico* analysis.

Objective 2:

To analyze the effect of damaging nsSNPs on ULK1 stability.

Objective 3:

To optimize T-ARMS-PCR and PCR-RFLP assays for earlier reported SNPs and predicted nsSNPs.

Objective 4:

Genotyping of SNPs and their association with HBV infection risk.

3. Materials and methods

3.1 Study subjects and sample collection

The studied population represents 700 subjects of North Indian region.The median age is 35 ±14 years for HBV infected patients, and 37±13 years for control subjects.Out of these total participants, 500 are HBV infected patients, which are enrolled in the study from the period of 3 years from 2013-2016, and 200 healthy controls. Diagnosis of hepatitis B was based on the presence of Hepatitis B surface antigen (HBsAg), anti-HBs, anti-HBC total, HBeAg, anti HBe, aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and HBV DNA levels. The basic demographic data for all the patients were recorded and they or their legal representatives gave their consent for the study. Chronic HBV infection was diagnosed by detection of HBsAg over a period of 6 months. The blood samples from patients and healthy controls were collected in sterile, vacutainers (Red cap for *serum* and Purple cap for *DNA* isolation) and transferred to the laboratory under proper cold chain conditions for further processing.

3.2 Processing of blood samples

The blood samples collected in red cap vacutainers were used for serum separation. The red cap vacutainers were centrifuged at 2500 rpm for 10 minutes for better separation. Whole genomic DNA was isolated from the purple cap vacutainers containing Ethylenediaminetetraacetic acid (EDTA) as an anti-coagulating agent using standard salting out the procedure described by Miller *et al.*[28] and also the DNA aliquots were stored at - 20 C.

3.3 Liver function tests

The biochemical estimations(Serum glutamic oxaloacetic transaminase and serum glutamate pyruvate transaminase) in serum were carried out using Erba diagnostic kits(ERBA diagnostics, Germany).

3.4 Quantification of HBV DNA

The HBV DNA from patients were isolated using QIAamp DNA Blood Mini Kit (QIAGEN Inc, Valencia, CA). Quantitative RT-PCR was then used for the identification of HBV DNA copies present in the serum of patients.

3.5 Data set for *in-silico* analysis

The nsSNPs are the nucleotide changes that result in an altered amino acid in the protein sequence. This altered amino acid may or may not affect the function of the protein. The affected protein function in case of modifying nsSNPs is due to change in its (i) structure, (ii) stability and (iii) by influencing functional binding sites. We have used Sorting Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen) tools to identify nsSNPs, which are popular standard tools to predict intolerant or damaging variants.

3.6 Mutation-based sequence stability analysis

IPTREE-STAB and i-Mutant2.0 tools were used to predict the change in protein stability on encountering mutations. iPTREE-STAB is a sequence-based web server that predicts stability change upon single amino acid substitution[29]. Whereas, i-Mutant2.0 tool is a support vector machine (SVM)-based tool for the prediction of change in protein stability in terms of $\Delta\Delta G$ values [30].

3.7 Mutation-based structure stability analysis

One nsSNP i.e. rs79965940 (N148T) out of the four nsSNPs was mapped on the partial available protein structure of ULK1 (PDB ID: 4WNO). As there was no PDB structure available for the complete sequence of ULK1, we used Robetta (Rosetta Comparative Modeling and *Ab Initio* Modeling) web-based server (http://robetta.bakerlab.org) for the prediction of full-chain protein structure by comparative modeling [31]. The second nsSNP i.e.rs61942435 (A991V), was mapped onto the structure (ULK1 modeled structure A-ULK1A) obtained from Robetta. Further, DUET and ERIS tools were used for predicting the protein stability change incurred upon mutations[32, 33].

3.8 Prediction of total energy for native and mutant structures

Mutant structures for the mutations N148T in 4WNO and A991V in ULK1A were generated using PyMOL (Molecular Graphics System, Version 1.8 Schrödinger, LLC)[34]. These mutant structures (N148T in 4WNO and A991V in ULK1A), as well as their native structures (4WNO and ULK1A), were submitted for carrying out energy minimizations and total energy computation using CHARMMforcefield (Discovery Studio 4.1).

3.9 Genotype analysis of SNPs in ULK1 gene

Non-synonymous polymorphisms and other polymorphisms which are reported previously for their role in Crohn's disease[26, 27], ankylosing spondylitis[35] and non-small cell lung cancer[36] are further validated in this study for their association with HBV infection. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping 4nsSNPsand rs3923716, whereas the genotyping of rs12303764 was performed by newly developed T-ARMS PCR assay.The PCR was carried out in a final volume of 25 µl containing 20 ng of genomic DNA, 0.2 mM of each dNTP, 5mM MgCl2, 50mM NaCl, 50mM Tris-HCl (pH 9.0), 10µg activated calf thymus DNA; 0.1mg/ml BSA. For the restriction digestion analysis of rs79965940 (N148T), rs61942435 (A991V), rs55824543 (T503M) and rs56364352 (S298L), 10ul aliquots of the PCR products were digested overnight in 1unit of *Mme*I, *Hin*dIII, *Nsp*I and *Bbv*I restriction enzymes (New England Biolabs, UK Ltd.) respectively. The digested PCR product was ultimately separated by electrophoresis in 2% agarose gel and the fragments were visualized by UV transilluminator. A standard negative control was included in each PCR reaction.

3.10 Statistical analysis

Genotypes and the alleles frequencies were estimated by direct counting. The genotype frequencies were assessed for Hardy-Weinberg equilibrium with chi-square ($\chi 2$) test. Quantitative variables were compared using independent student's *t*-test. The results were expressed as odds ratio (OR), 95% confidence intervals (95% CI) and *p*-values. In the study, a *p*-value <0.05 is considered significant.All the statistical analyses and evaluations were performed using SPSS v.17.0 for Windows.

3.11 Haplotype analysis

Haplotype analysis was conducted to examine the genetic association of ULK1 with hepatitis B infection. These haplotypes were analyzed using SNPSTATS software (http: //bioinfo. iconlogica.net).

4. Results

4.1 *In-silico* mutational analysis

4.1.1 Change in the protein stability upon mutations at sequence level

Protein stability change was predicted by iPTREE-STAB and I-MUTANT2.0 in terms of Gibbs free energy change(ΔΔG) values as shown in **Table 1**. The ΔΔG value less than 0 signifies a decrease in protein stability while ΔΔG value more than 0 signifies an increase in protein stability. The results by iPTREE-STAB suggest that the three mutations (N148T, T503M, and S298L) decrease the protein stability while the mutation A991V increases the protein stability. Whereas, the results from i-MUTANT2.0 suggests that all the four mutations (N148T, A991V, T503M, and S298L) decrease the protein stability.

Table 1. Prediction of protein stability change upon mutations in terms of Gibbs free energy from protein sequence by using iPTREE-STAB and i-MUTANT2.0

ULK1 nsSNP	Amino acid substitution	iPTREE-STAB ΔΔG Value (kcal/mol)	Stability prediction	IMUTANT2.0 ΔΔG Value (kcal/mol)	Stability prediction
rs79965940	N148T	-0.79	Decrease	-0.74	Decrease
rs61942435	A991V	0.9422	Increase	-0.88	Decrease
rs55824543	T503M	-1.0376	Decrease	-0.21	Decrease
rs56364352	S298L	-1.1536	Decrease	-0.23	Decrease
ΔΔG <0: Decrease Stability; ΔΔG >0: Increase Stability					

4.1.2 Change in protein stability upon mutations at structure level

The protein stability change at structure level was predicted by DUET and ERIS tools in terms of Gibbs free energy change (ΔΔG) values as shown in **Table 2**. The ΔΔG value less than 0 signifies a decrease in protein stability while ΔΔG value more than 0 denotes an increase in protein stability for DUET and for ERIS tool, ΔΔG value less than 0 signifies an increase in protein stability while ΔΔG value more than 0 signifies a decrease in protein stability. The results by DUET indicate that both mutations (N148T and A991V) decrease the protein stability while the results from ERIS suggest that the mutation N148T decreases the protein stability and the mutation A991V increases the protein stability.

Table 2. Prediction of protein stability change upon mutations in terms of Gibbs free energy from protein structure by using DUET and ERIS

ULK1 nsSNP	ULK1 structure	Amino acid substitution	DUET ΔΔG Value (kcal/mol)	Stability prediction	ERIS ΔΔG Value (kcal/mol)	Stability prediction
rs79965940	4WNO	N148T	-0.372	Decrease	2.89	Decrease
rs61942435	ULK1A*	A991V	-0.246	Decrease	-1.16	Increase

4.1.3 Total energy of the protein upon mutations

Mutant structures for the mutations N148T in 4WNO and A991V in ULK1A were generated using PyMOL (Molecular Graphics System, Version 1.8 Schrödinger, LLC) and were represented in **Figure 1A** and **Figure 1B**. Energy minimizations and total energies were computed using CHARMM forcefield (Discovery Studio 4.1) and results are shown in **Table 3**. The total energy of native and mutant structures was compared and from the results, it was observed that the mutation N148T increased the total energy of the structure and hence could be considered as a destabilizing mutation. Whereas, the mutation A991V was found to decrease the total energy of the structure and therefore it could increase the stability of the protein.

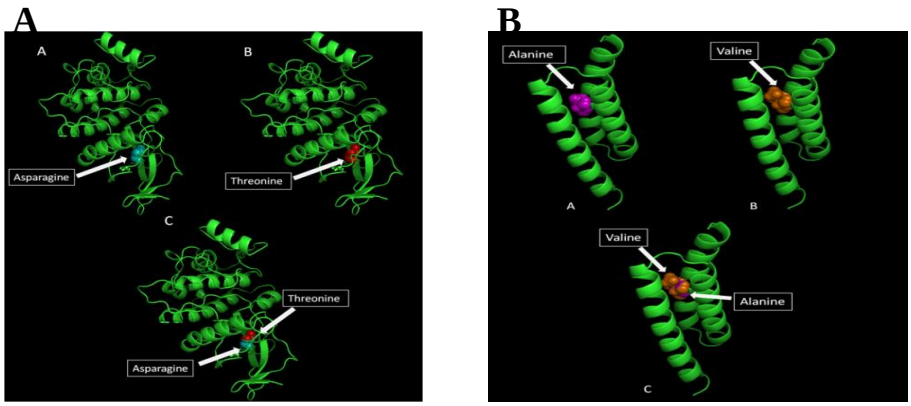

Figure1A. Structural representation of nsSNP rs79965940 (N148T) on the PDB structure of ULK1 (4WNO).(A) Native structure 4WNO with amino acid Asparagine at position 148 (B).Mutant structure N148T 4WNO with amino acid substitution as Threonine at position 148. (C). Superimposed native (4WNO) and mutant structures (N148T 4WNO). All structures were generated using PyMol. **B**: Structural representation of nsSNP rs61942435 (A991V) on the modeled structure of ULK1 (ULK1A). (A) Native structure ULK1A with amino acid Alanine at position 991 (B).Mutant structure A991V ULK1 with amino acid substitution as Valine at position 991. (C). Superimposed native (ULK1A) and mutant structures (A991V ULK1A). All structures were generated using PyMol.

*Full chain structure of ULK1 predicted and modeled by Robetta wherein ΔΔG <0: Decrease Stability; ΔΔG >0: Increase Stability (For DUET) and ΔΔG <0: Increase Stability; ΔΔG >0: Decrease Stability (For ERIS)

Table 3. Computation of total energy (CHARMm energy) of the native and mutant structures for mutations N148T and A991V in structures 4WNO and ULK1A predicted by CHARMm force field

S. No.	Structure	Force field	Potential energy/CHARMm energy (Kcal/mol)	Vander Waals energy (Kcal/mole)	Electrostatic energy (Kcal/mole)	Final RMS gradient (Kcal/mole X A^0)
Native	4WNO	CHARMm	-23179.83	-2145.89	-23558.82	157.4
N148T	4WNO	CHARMm	-23112.05	-2097.40	-23652.33	112.9
Native	ULK1A*	CHARMm	-4259.87	-452.66	-4320.24	24.1
A991V	ULK1A*	CHARMm	-4268.66	-449.41	-4346.36	24
* Full chain structure of ULK1 predicted and modeled by Robetta						

4.2 Study subjects

The detail of the base-line biochemical and demographic characteristics of the patients investigated in this study are described in **Table 4.**

Table 4. Base-line biochemical and demographic characteristics of patients

Characteristic	Patients Characteristic	Control subjects
Gender (male/female)	314/186	110/90
Age [Mean ± SD]	35±14	37±13
History of alcohol consumption		
Yes	296	20
No	204	180
HBV DNA (Mean log copies/ml ± SD)	8.20± 9.45	-ve
HBsAg	+ve	-ve
HCV	-ve	-ve
HIV	-ve	-ve
ALT (Mean ± SD)	89.10±86.35	33.4±13.4
AST (Mean ± SD)	87.95±72.88	25.6±8.8
Clinical diagnosis		
Asymptomatic carrier	142	-ve
Acute Hepatitis	114	-ve
Chronic Hepatitis	207	-ve
Liver cirrhosis	37	-ve

4.3 Liver function tests (LFT)

Collected serum of 500 patients was used for analysis of LFT for enzyme aspartate aminotransferase (AST), also known as serum glutamic oxaloacetic transaminase (SGOT) and alanine aminotransferase (ALT), also known as serum glutamic pyruvic transaminase (SGPT) (**Figure 2**). These biochemical values were further used for differentiating patients into various clinical groups.

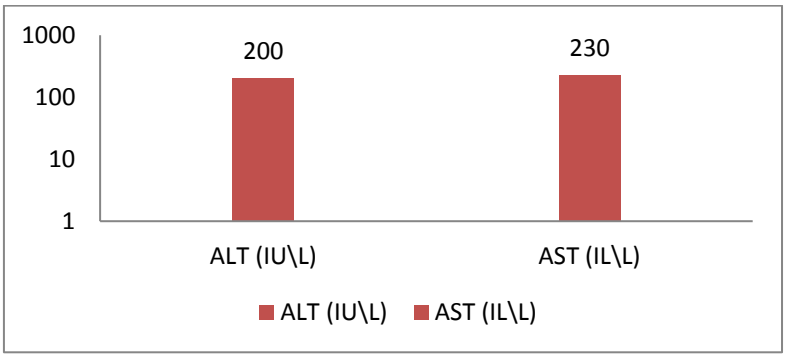

Figure 2. Liver function tests levels in Hepatitis B infected patients.

4.4 Quantification and grouping of HBV infected patients

HBV DNA from 500 patients was isolated and subjected to quantification of viral load by Quantitative RT-PCR. As seen in **Figure 3**, there was a significant increase in HBV DNA levels with respect to the severity of HBV infection among patients.

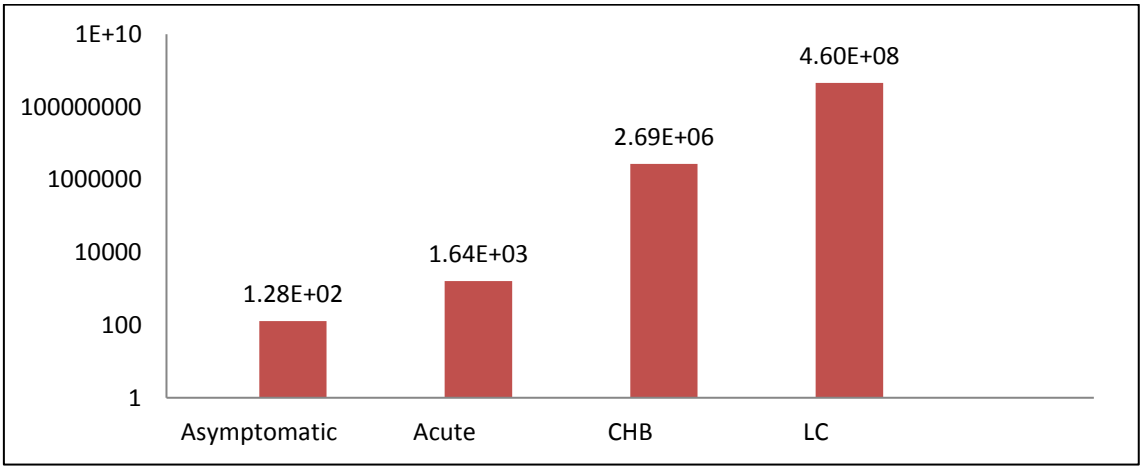

Figure 3.HBV DNA levels in Hepatitis B infected patients.

4.5 Genetic correlation of nsSNPs in Hepatitis B patients and healthy controls

The PCR-RFLP and TARMS-PCR banding pattern of four nsSNPs in ULK1 are portrayed in **Figure 4**. The genotype and allele frequencies of four nsSNPs of ULK1 in HBV patients and healthy controls are shown in **Table 5**. Interestingly, two genetic variants have shown statistically significant differences between HBV patients and controls. The most significant result was observed for rs56364352 showing that the C allele was present in 60.4% of patients and 72% of the controls depicting a significant association with HBV infection (OR-1.69; 95% CI-1.30 to 2.17; p- <0.01). Similarly, rs79965940 has also shown significant difference with A allele present in 67% of the patients and 74% of the controls, showing an association of this SNP with HBV infection (OR -1.40; 95% CI-1.08 to 1.81; p-0.01). For the remaining two polymorphisms we didn't observe any statistically significant results between the patients and the controls.

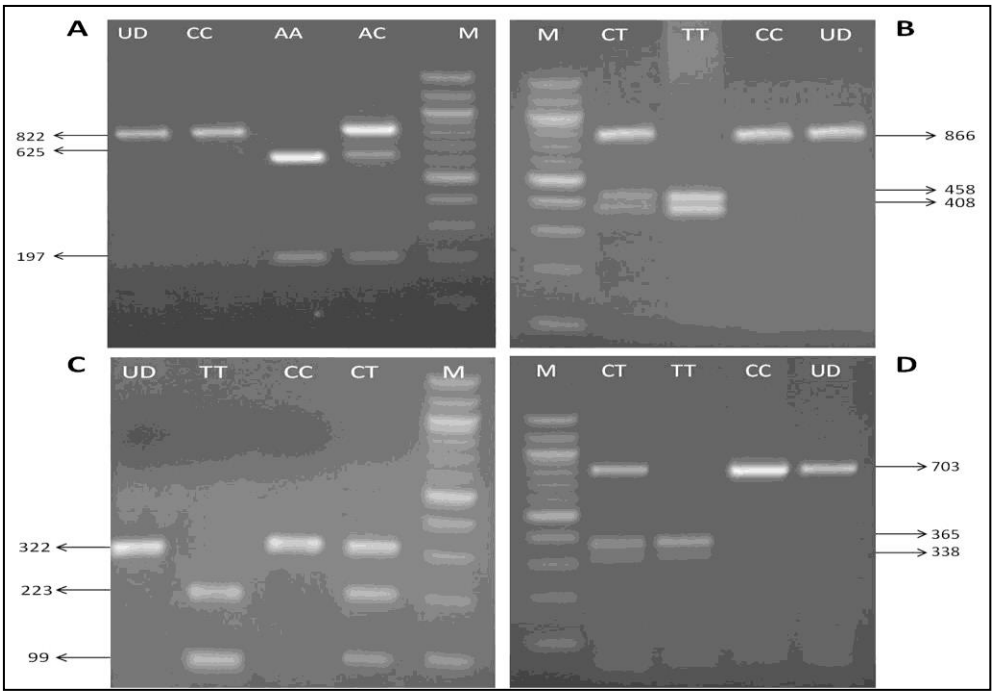

Figure 4.PCR-Restriction Fragment Length Polymorphism Analysis of nsSNPs ofULK1 Gene.
A-rs79965940 (A/C), UD- undigested; CC-822bp;AA-625bp,197bp;AC- 822bp,625bp and 197bp
B- rs56364352 (C/T), UD- undigested; CC-866bp; TT- 458bp and 408bp; CT- 866bp,458bp and408bp
C- rs61942435 (C/T), UD- undigested; CC-322bp; TT- 223bp and 99bp; CT- 322bp,223bp and 99bp
D- rs55824543 (C/T), UD- undigested; CC-703bp; TT- 365bp and 338bp; CT- 703bp,365bp and 338bp

ULK1 nsSNPs	Patient (%) (n = 500)	Normal (%) (n = 200)	Allele	HBV	Control	P value	Logistic Regression; OR	95% CI
rs79965940 (A/C)								
AA	260 (52)	118 (59)	A allele	670 (67%)	296 (74%)		1.00[Ref]	
AC	150 (30)	60 (30)	C allele	330 (33%)	104 (26%)	**0.01**	**1.4018**	**1.08-1.81**
CC	90 (18)	22 (11)						
rs61942435 (C/T)								
CC	300 (60)	114 (57)	C allele	775(77.5 %)	302(75.5)		1.00[Ref]	
CT	175 (35)	74 (37)	T allele	225(22.5%)	98(24.5%)	0.42	0.89	0.68-1.17
TT	25 (5)	12 (6)						
rs56364352 (C/T)								
CC	197 (39.4)	110 (55)	C allele	604 (60.4%)	288 (72%)	1.00[Ref]		
CT	210 (42)	68 (34)	T allele	396 (39.6%)	112 (28%)	**<0.01**	**1.68**	**1.30-2.16**
TT	93 (18.6)	22 (11)						
rs55824543 (C/T)								

CC	350 (70)	130 (65)	C allele	796 (79.6%)	306 (76.5%		1.00[Ref]	
CT	96 (19.2)	46 (23)	T allele	204 (20.4%)	94 (23.5%)	0.20	0.83	0.63-1.10
TT	54 (10.8)	24 (12)						

Table 5. The genotype and allele frequencies of four nsSNPs of ULK1 in HBV patients and healthy control

Similarly, when various genetic models were used to find a significant association in the selected SNPs with HBV patients and healthy controls it was observed thatrs79965940 confirm a significant association with HBV infection (OR-1.77; 95% CI-1.07 to 2.92; p- 0.02) in the recessive model. This analysis was in a strong support of the above results. Likewise, rs56364352 also confirms noteworthy association with HBV infection in recessive (OR-1.84; 95% CI-1.12 to 3.03; p- 0.015) and dominant (OR-1.87; 95% CI-1.34 to 2.61; p<0.02) models. Apart from these, rs61942435 and rs55824543 didn't show any association with HBV infected patients in any of the genetic model included in this study.

4.6 Genetic correlation of nsSNPs in hepatitis B patients with different clinical groups

The study patients were broadly stratified into four sub-clinical categories based on the severity of HBV infection, including asymptomatic, acute, chronic Hepatitis B and liver cirrhosis. When these clinical groups were compared to healthy individuals, regression analysis for ULK1 nsSNPs rs79965940, rs61942435, rs55824543 and rs56364352 provided supportive and remarkable outcome which support our above-mentioned results. As shown in **Table 6**, nsSNP rs56364352 was found to be significantly associated with three sub-clinical categories, i.e. asymptomatic patients group (OR-1.72; 95% CI-1.24 to 2.38; p- <0.01), acute patients group (OR- 1.97; 95% CI-1.40 to 2.77; p- <0.01) and chronic Hepatitis B patients group (OR- 1.70; 95% CI-1.27 to 2.28; p- <0.01), but no such association was seen with liver cirrhosis patients group. Similarly,nsSNP rs79965940 also showed a significant association with two sub-clinical categories, i.e. acute patients group (OR 1.53; 95% CI-1.08 to 2.18; p-0.01) and chronic Hepatitis B patients group (OR- 1.40; 95% CI-1.03 to 1.90; p-0.02), but no such association was seen with asymptomatic patients group and liver cirrhosis patients group. No significant difference was observed among nsSNPs rs61942435 and rs55824543 when compared with sub-clinical groups, suggesting no association with the severity of HBV infection.

4.7 Genotypes and alleles frequencies of rs12303764 and rs3923716

Figure 5 represents the PCR-RFLP and T-ARMS-PCR analysis of rs12303764 and rs3923716. Out of the 700 subjects, as shown in **Table 7**, ULK1 rs12303764 genotype TT was present in 207(41.2%), GT was present in 229(45.80%), and GG was present in 64 (12.8%). The ULK1 rs3923716G\T genotype GG was present in 280subjects (56.00%), GT was present in 160 (32.00%) and TT was present in 60 (12.00%). In both the SNPs, all the genotypes were found in patients as well as controls. For rs12303764 the most frequent genotype in HBV patients was GT 45.8% (229/500) where as the most frequent in healthy controls was TT 60% (120/200). Similarly, in ULK1 rs3923716, all the genotypes were detected in both cases and controls. The mutant TT genotype was found in 12% (60/500) of the HBV patients and 6.5% (13/200) controls. The proportion of GT genotypes was 47.5% (160/500) and 30% 60/200 in the HBV patients and controls, respectively. The most frequently identified genotype was GG; 56% (280/500) in HBV patients and 63.5% (127/200) in healthy controls. From **Table 7** it is observed that mutant T allele of rs3923716G\T is associated with HBV infection (OR- 1.41; 95% CI - 1.07-1.87;p - 0.01), whereas no such association is seen with rs12303764 G\T (OR- 0.64, 95% CI- 0.49-0.82;p - <0.05).

Table 6. Association analysis of two SNPs with clinical groups

SNP	Clinical Group	OR (95% CI)	P-value
rs79965940 (A/C)	Asymptomatic (142)	1.32 (0.94-1.84)	0.10
	Acute Hepatitis (114)	**1.53 (1.08-2.18)**	**0.01**
	Chronic Hepatitis (207)	**1.40 (1.03-1.90)**	**0.02**
	Liver cirrhosis (37)	1.28 (0.74-2.20)	0.36
rs61942435 (C/T)	Asymptomatic (142)	0.93 (0.65-1.33)	0.70
	Acute Hepatitis (114)	0.95 (0.65-1.39)	0.81
	Chronic Hepatitis (207)	0.94 (0.68-1.30)	0.72
	Liver cirrhosis (37)	0.37 (0.17-0.80)	0.01
rs55824543 (C/T)	Asymptomatic (142)	0.78 (0.53-1.13)	0.19
	Acute Hepatitis (114)	1.05 (0.72-1.54)	0.76
	Chronic Hepatitis (207)	0.79 (0.56-1.10)	0.17
	Liver cirrhosis (37)	0.63 (0.32-1.21)	0.07
rs56364352 (C/T)	Asymptomatic (142)	**1.72 (1.2-2.3)**	**<0.01**
	Acute Hepatitis (114)	**1.97 (1.40-2.77)**	**<0.01**
	Chronic Hepatitis (207)	**1.70 (1.27-2.28)**	**<0.01**
	Liver cirrhosis (37)	0.82 (0.46-1.46)	0.51

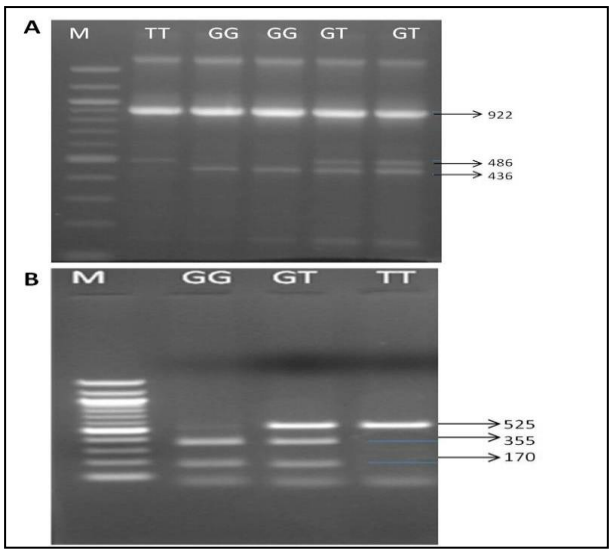

Figure 5.T-ARMS-PCR and PCR-RFLP analysis of rs12303764 and rs3923716.
A-rs12303764; M-100bp DNA ladder; TT-922bp and 486bp; GG- 922bp and 436bp; GT- 922bp,486bp and 436bp. **B**- rs3923716; M-100bp DNA ladder; GG-355bp and 170bp; GT- 525bp, 355bp and 170bp; TT- 525bp.

Table 7. Genotype and allele frequencies of ULK1 rs12303764G\T and rs3923716G\T polymorphism in HBV patients and healthy controls

SNP	Genotype	HBV N=500 (%)	Control N=200 (%)	Allele	HBV (%)	Control (%)	OR (95% CI)	P value
rs12303764 G\T	T/T	207 (41.2)	120 (60)	T	643 (64.3)	295 (73.7)	Referent	
	G/T	229 (45.8)	55 (27.5)	G	357 (35.7)	105 (26.3)	0.64 (0.49–0.82)	<0.05
	G/G	64 (12.8)	25 (12.5)					
rs3923716 G\T	G/G	280 (56)	127 (63.5)	G	720 (72)	314 (78.5)	Referent	
	G/T	160 (32)	60 (30)	T	280 (28)	86 (21.5)	**1.41 (1.07–1.87)** 0.01	
	T/T	60 (12)	13 (6.5)					

4.8 Correlation of ULK1 rs12303764G\T and rs3923716G\T polymorphisms with clinical variables

A total of 500 Hepatitis B patients were divided into four groups based on their clinical diagnosis. Out of these 500 patients, 142 were included in asymptomatic, 114 in acute hepatitis, 212 in Chronic Hepatitis and 32 in liver cirrhosis category. The association analysis

of two SNPs with asymptomatic, acute hepatitis, chronic hepatitis and liver cirrhosis is shown in **Table 8**.

Table 8. Association analysis of two SNPs with clinical groups

SNP	Clinical Group	OR (95% CI)	P value
rs12303764 G\T	Asymptomatic (142)	0.79 (0.56-0.10)	0.17
	Acute Hepatitis (114)	0.51 (0.36-0.72)	0.0002
	Chronic Hepatitis (207)	0.59 (0.44-0.79)	0.0006
	Liver cirrhosis (37)	0.98 (0.54-1.78)	0.9579
rs3923716 G\T	Asymptomatic (142)	**1.48 (1.04-2.10)**	**0.027**
	Acute Hepatitis (114)	1.27 (0.87-1.86)	0.21
	Chronic Hepatitis (207)	0.20 (0.11-0.34)	0.0001
	Liver cirrhosis (37)	**1.91 (1.08-3.37)**	**0.025**

Out of the two SNPs, mutant T allele of rs3923716 G\T is associated with asymptomatic infection (P = 0.027, OR = 1.48, 95% CI= 1.04-2.10) and liver cirrhosis (P = 0.025, OR= 1.91, 95% CI= 1.08-3.37) groups only, while there was no significant association observed between rs12303764 G\T and any of the four sub groups of HBV infected patients. However, rs3923716was associated with an increased probability of HBV infection (Co-dominant model: P= 0.0043, OR= 1.61, 95% CI=1.10-2.35; Over-dominant model: P= 0.0052, OR= 1.71, 95%CI=1.18-2.49).On the contrary, rs12303764 G\T was not associated with any of the genetic models.

4.9 Correlation of HBV DNA viral load with genotypes of polymorphisms in ULK1 gene

After quantifying viral load in different categories of HBV patients, we further correlated the HBV DNA levels with different genotypes in selected polymorphisms of ULK1 gene (**Figure**

6).

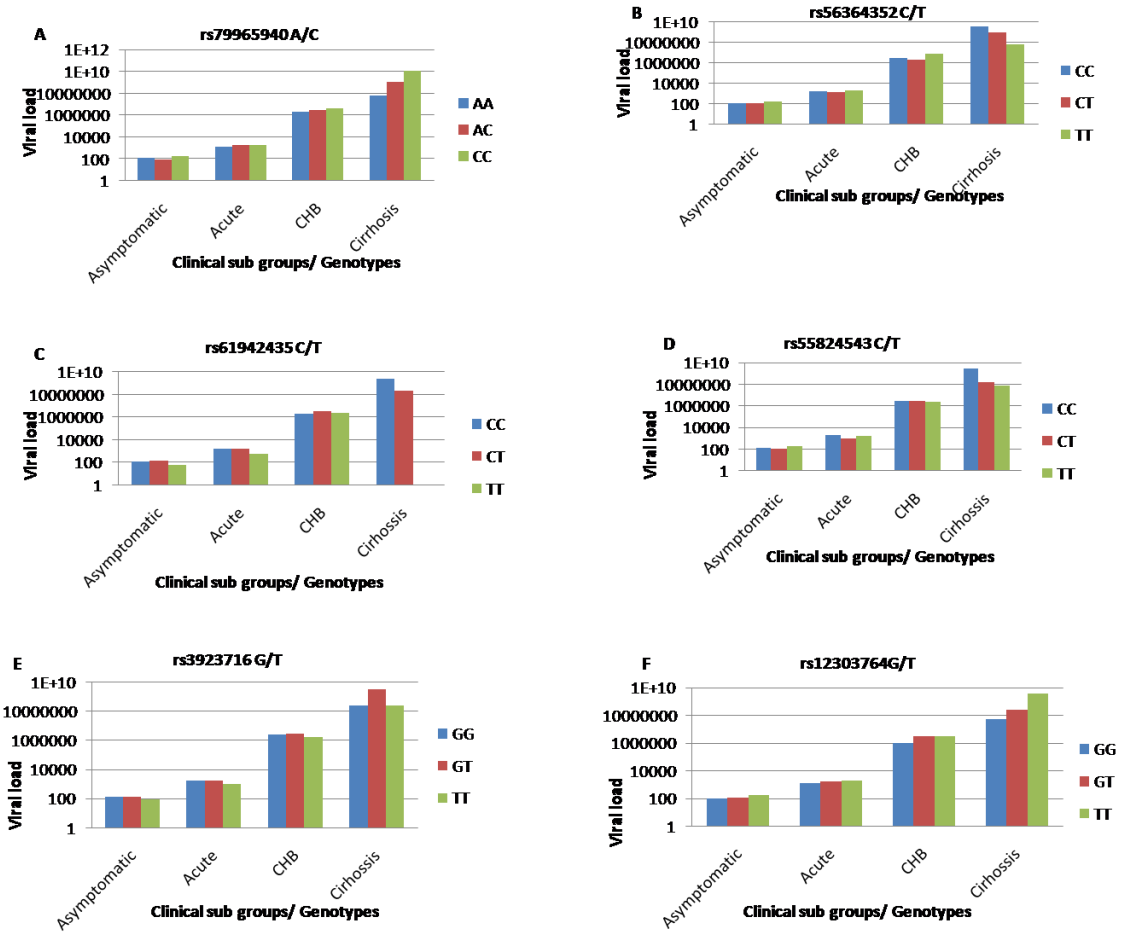

Figure 6. Correlation between genotypes of 6 polymorphisms of ULK1 gene with a viral load of different HBV clinical categories.

- rs79965940 A/C: We have correlated the viral load of different categories of HBV patients with three genotypes (AA, AC, and CC) of non-synonymous polymorphism rs79965940A/C, results are shown in **Figure 6A**. On analyzing the data it was found that the mutant CC genotype is correlated with an increased viral load in all the clinical categories of HBV patients. The results we obtained here clearly indicate and support our previous discussed results (**Table 5** and **6**) that CC genotype and C allele of rs79965940 was found to increase the disease risk in North Indian population.

- rs56364352C/T: On analyzing the data it was found that the mutant TT genotype of rs56364352 C/T is correlated with an increase of viral load in asymptomatic, acute and chronic categories of HBV patients (shown in **Figure 6B**). The results hence obtained

clearly indicate and support our previous discussed results (**Table 5** and **6**) that the TT genotype and T allele of rs79965940 were found to increase the disease risk in North Indian population.

- rs61942435 C/T: A correlation between the viral load of different HBV categories with three genotypes (CC, CT, and TT) of rs61942435 C/T was analyzed, as shown in **Figure 6C**. On examining the data it was found that the mutant TT genotype did not show any correlation with increased viral load among different clinical categories of HBV patients. The results we obtained here clearly indicate that there is no significant association of TT genotype and T allele of rs79965940 with increased disease risk in North Indian population.

- rs55824543 C/T: We have also correlated the viral load of different categories of HBV patients with three genotypes (CC, CT, and TT) of rs55824543 C/T, results shown in **Figure 6D**. On analyzing the data it was found that the mutant TT genotype did not show any correlation with increased viral load among different clinical categories of HBV patients. The results we obtained here clearly indicate that there is no significant association of TT genotype and T allele of rs55824543 C/T with increased disease risk in North Indian population.

- rs3923716 G/T: On analyzing the data it was found that the mutant TT genotype did not show any correlation with increased viral load among different clinical categories of HBV patients (results shown in **Figure 6D**). The results we obtained here clearly indicate that there is no significant association of TT genotype and T allele of rs79965940with increased disease risk in North Indian population.

- rs12303764 G/T: We correlated the viral load of different categories of HBV patients with three genotypes (GG, GT, and TT) of rs12303764 G/T, results shown in **Figure 6F**. On analyzing the data it was found that the mutant TT genotype did not show any correlation with increased viral load among different clinical categories of HBV patients. The results we obtained here clearly indicate that there is no significant association of TT genotype and T allele of rs12303764G/T with increased disease risk in North Indian population.

4.10 Relation between ULK1 gene polymorphisms and biochemical parameters

Statistical analysis on the correlation between genotypes of polymorphisms in ULK1 gene and clinical features of HBV patients were further evaluated. As shown in **Table 9**, the detailed analyses exhibited that AC or CC genotype of rs79965940 A/C had significantly increased serum ALT, AST levels at presentation than those with AA genotype ($p < 0.0001$ for both). Besides, there was no significant difference between the total bilirubin level and genotypes of rs79965940 A/C. Similarly, there is no significant difference between other polymorphisms of ULK1 gene and clinical features of HBV patients.

Table 9.Association of ULK1 polymorphism with clinical features of HBV patients

Clinical Features	**ULK1 Polymorphism**		
	rs79965940 A/C		
	AA	AC+CC	P-value
ALT(IU/L)	70±60	110±104	**< 0.0001**
AST(IU/L)	75±48	102±91	**< 0.0001**
TBIL (μmol/L)	0.87±0.46	0.89±0.46	0.6274
	rs61942435 C/T		
	CC	CT+TT	P value
ALT(IU/L)	89±83	91±89	0.7035
AST(IU/L)	88±74	88±72	1.0000
TBIL (μmol/L)	0.88±0.44	0.89±0.50	1.0000
	rs55824543 C/T		
	CC	CT+TT	P value
ALT(IU/L)	87±65	94±122	0.4052
AST(IU/L)	86±62	93±94	0.3267
TBIL (μmol/L)	0.90±0.47	0.82±0.42	0.0726
	rs56364352 C/T		
	CC	CT+TT	P value
ALT(IU/L)	83±60	93±100	0.2071
AST(IU/L)	87±54	89±83	0.76
TBIL (μmol/L)	0.83±0.44	0.91±0.47	0.06
	rs3923716 G/T		
	GG	GT+TT	P value
ALT(IU/L)	87±84	92±89	0.5202
AST(IU/L)	86±68	91±78	0.4448
TBIL (μmol/L)	0.86±0.47	0.90±0.45	0.3363
	rs12303764G/T		
	GG	GT+TT	P value
ALT(IU/L)	82±93	90±85	0.4877
AST(IU/L)	85±63	88±74	0.758
TBIL (μmol/L)	0.94±0.41	0.87±0.47	0.2591

4.11 Haplotype analysis of nsSNPs of ULK1

Moreover, when haplotype analysis was performed in context of SNPs in ULK1,one high-risk haplotype, ATCC (OR- 1.74; 95% CI- 1.03 to 2.94; P=0.03)illustrated the relationship with susceptibility/ clinical presentation of HBV, as shown in **Table 10**.We found only two samples with a haplotype sequence of ATCC in our samples. Also for the first time, a correlation of haplotype (ULK1-ATCC) with HBV infection was reported. Importantly, SNP variation at rs61942435 C→T seems to influence HBV presentation.

Table 10. Haplotype analysis of four nsSNPs of ULK1

S.No	rs79965940 A/T	rs61942435 C/T	rs55824543 C/T	rs56364352 C/T	Frequency	OR (95%CI)	P-value
1	A	C	C	C	0.2629	1.00	-
2	A	C	C	T	0.1593	0.75 (0.48-1.16)	0.2
3	C	C	C	C	0.1224	1.16(0.75-1.79)	0.52
4	**A**	**T**	**C**	**C**	**0.0894**	**1.74 (1.03-2.94)**	**0.039**
5	A	C	T	C	0.0686	1.42 (0.83-2.43)	0.2
6	C	C	C	T	0.059	0.57 (0.27-1.19)	0.14
7	A	T	C	T	0.042	0.70 (0.30-1.65)	0.41
8	C	C	T	C	0.0389	0.67 (0.31-1.46)	0.31
9	A	C	T	T	0.0352	0.69 (0.31-1.57)	0.38
10	C	T	C	T	0.032	0.48 (0.16-1.41)	0.18
11	A	T	T	C	0.0237	1.46 (0.60-3.55)	0.41
12	C	C	T	T	0.023	0.90 (0.35-2.28)	0.82

5. Discussion

The major objective in the field of human genetics is to identify mutations that may act as biomarkers for disease susceptibility.GWAS and immunogenetic studies have already provided great insights into the pathogenesis of HBV and its relation to various autophagy-related genes. Recent studies showed that susceptibility to HBV infection with the potential of destructive liver inflammation is based on host genetic factors that may predispose to or protect from disease [37-39]. Hence, autophagy alone may not necessarily participate in HBV infection, but it may provoke disease in the context of other risk variables, such as host genotype, aging,

and/or behavioral habits such as alcohol intake. Previous studies have also demonstrated that increased/decreased autophagy function may promote/suppress the development of HBV [40]. In this way, variable levels of autophagy can lead to HBV progression; causing liver damage in susceptible subjects [41]. Although the genetic basis of autophagy has been thoroughly acknowledged with its role associated to HBV, but still genetic variants such as of ULK1 has not been examined with context to HBV infection. Owing to these facts, the current study investigated whether nsSNPs rs61942435, rs79965940, rs55824543 and rs56364352and SNPs rs12303764 and rs3923716 in ULK1 gene might be linked with the susceptibility to HBV infection and its clinical sub-groups in NorthIndian population.

This investigation is the first explorative approach focusing on the relation between genetic variants of ULK1 and HBV. We predicted four nsSNPs (rs61942435, rs79965940, rs55824543 and rs56364352) [42] and further analyzed their impact on protein stability and genotyped these polymorphisms for understanding their role in HBV infection. The results from *in-silico* sequence-based analysis suggests that all the nsSNPs (rs61942435, rs79965940, rs55824543 and rs56364352) decreased the protein stability as predicted by I-MUTANT2.0; while one of them i.e. (rs61942435) was found to increase the protein stability as predicted by iPTREE-STAB. Furthermore, thesensSNPswere mapped on the ULK1 protein structures (4WNO [rs79965940] and ULK1A [rs61942435]) to predict the protein stability change at the structural level. According to DUET prediction, both the nsSNPs (rs79965940 and rs61942435) decrease the protein structural stability whereas; the nsSNPrs61942435 is found to increase the protein stability according to the ERIS tool. In order to predict the total energy of the protein structure, we used Discovery Studio to compute the total energy with CHARMm force field. It was found that rs79965940 nsSNP (N148T) increased the total energy of the protein structure and hence could be regarded as a destabilizing mutation. On the other hand, the nsSNP rs61942435 (A991V) decreased the total energy and hence could be regarded as stabilizing mutation. Overall, the nsSNP rs61942435 was found to increase the protein stability while, the nsSNPs rs79965940, rs55824543, and rs56364352 were found to decrease the protein stability both at sequence and structure level. Therefore, we selected these four nsSNPs for genotyping in the HBV patient samples for determining their role in disease susceptibility or progression.

Using a large, well-characterized North Indian population group, the foremost findings reported here with respect to nsSNPs of ULK1 was that, although all the genotype variants

were detected both in the patients and controls but rs79965940 and rs56364352 were independently associated with HBV infection whereas nsSNPs rs61942435 and rs55824543 were not associated with HBV infection respectively.The statistically significant association shown by rs79965940 (OR-1.77; 95% CI-1.07 to 2.92; P - 0.02) and rs56364352 (OR-1.69; 95% CI-1.30 to 2.17; P<0.01) has revealed the importance of these polymorphisms with susceptibility to HBV infection. Further, we assessed if these 4 nsSNPs have any association with sub-clinical HBV groups. Interestingly our results revealed a substantial association of ULK1 rs79965940 with two subclinical HBV groups i.e. acute and CHB risk groups. Similarly, rs56364352 have also shown noteworthy association with three sub-clinical HBV groups i.e. acute, asymptomatic and CHB risk group. In addition, we also noted that ULK1 rs79965940 C allele and CC genotype were both significantly associated with increased acute and CHB risk among North Indian population. Furthermore, the recessive model (combined AA and AC) was correlated with a considerable increase in HBV risk. Likewise, for rs56364352 we found that T allele and TT genotype were both considerably associated with increased asymptomatic, acute and CHB infection risk among North Indian population. Eventually, on analysis of mutant genotypes of rs56364352 and rs79965940, it was clearly observed that there was an increase in hepatitis B viral load as HBV infection progresses from asymptomatic infection to LC.

In our work, we have also investigated the possible connection of ULK1 SNPs rs3923716G/T and rs12303764G/T with HBV infection in North Indian population. Wherein the frequencies of GG genotype (56%) and G allele (72%) of rs3923716G\T were different in the HBV patients compared with those in healthy controls. The logistic regression analysis illustrated that the G allele of rs3923716G\T is associated with HBV infection (OR- 1.41; 95% CI- 1.0-1.87;p- 0.012). Further, an extensive analysis on different clinical groups of HBV infection depicts interesting results showing that G allele of rs3923716G\T is associated with asymptomatic infection (OR- 1.48; 95% CI- 1.04-2.10;p- 0.027) and liver cirrhosis (OR- 1.91; 95% CI- 1.08-3.37; p- 0.02) but not associated with acute hepatitis (OR- 1.27; 95% CI- 0.87-1.86;p- 0.21) and chronic hepatitis (OR- 0.20; 95% CI- 0.11-0.34;p- 0.0001). These results suggest that TT genotype and T allele of rs3923716G\T is involved in disease progression as this allele is maximally represented among patients that are either at the first stage of liver disease or at the last stage.Similarly, when mutant TT was compared with hepatitis B viral load among clinical sub-groups, it was observed that the viral load increases when HBV infection progresses from asymptomatic to LC infected patients.In addition, higher serum ALT, AST levels in HBV

patients were associated with rs79965940. In other words, the patients with rs3923716T/T genotype of ULK1 gene are more likely to have a progressive status of HBV infection.

6. Conclusion

This is the first study of its kind, presenting genetic association between polymorphisms of ULK1 gene with HBV infected patients. The study focuses on the prediction of nsSNPs of ULK1 gene and their susceptibility to HBV infection in North Indian Population. The results of this study demonstrate that mutant alleles of rs79965940 and rs56364352 destabilize the ULK1 protein structure. On further comprehensive analysis and assessment, it is now evident that rs79965940, rs56364352, and rs3923716 are associated with higher risk of HBV infection developing from initial asymptomatic infection to more severe chronic infection. Moreover, the identification of haplotype ATCC in the study is statistically associated HBV infection development. In conclusion, we anticipate that these analyzed SNPs can be used as genetic biomarkers and can act as a bridge between the research gap on autophagy and HBV relation.

7. References

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Publications

Journal Publications

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